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Cell cycle control of a Burkitt lymphoma cell line: responsiveness to growth signals engaging the C3D/EBV receptor

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SUMMARY

CR2, the receptor for the C3d fragment of the third complement component and for Epstein–Barr virus (EBV) has been shown, on mouse B cells, to be involved in the control of B-cell proliferation by acting as a receptor for macrophage-derived growth factors. We examined whether the growth of a Burkitt lymphoma cell line, RAJI, could be influenced by ligands of human CR2. In serum-free culture, purified human C3d, as well as three monoclonal antibodies to distinct epitopes on human CR2, were capable of enhancing the growth rate of RAJI cells two to five-fold. This effect could not be observed if even trace amounts of serum were present in the culture medium. Simultaneous addition of pairs of antibodies did not enhance the growth rate, suggesting that a particular engagement of CR2 may be critical in order to induce a stimulatory effect. These results indicate that in a homologous serum-free human B-cell system human C3d as well as monoclonal antibodies to human CR2 can induce B-cell proliferation and that CR2-mediated triggering of B cells can be induced via epitopes other than the C3d-binding site. In addition we conclude that—unlike normal human B cells—at least some human B-lymphoma cells respond to CR2-mediated stimuli in the absence of any T-cell derived factors. Therefore the control mechanisms exerted through CR2 must still be intact on these autonomously growing cells.

INTRODUCTION

The receptor for the C3d fragment of the third complement component, CR2, a glycoprotein of 145,000 MW (Barel, Charriaut & Frade, 1981; Weis, Tedder & Fearon, 1984; Micklem, Sim & Sim, 1984), has been shown recently to act also as the receptor for the Epstein–Barr virus (EBV) (Frade *et al.*, 1985a; Fingeroth *et al.*, 1984; Nemerow *et al.*, 1985b) and to be involved in the control of B-cell proliferation (Nemerow, McNaughton & Cooper, 1985a; Frade *et al.*, 1985b; Melchers *et al.*, 1985; Petzer *et al.*, 1988). In the case of murine B cells kept in serum-free culture it was shown that engagement of this receptor by cross-linked C3d enables the preactivated B cell to progress through the S-phase of its cycle. An additional T-cell-derived factor then provides the signal necessary for the beginning of mitosis (Melchers *et al.*, 1985, 1986). For human B cells it was also shown that stimulation of B cells via CR2 requires the presence of a T-cell-derived factor (Frade *et al.*, 1985b; Petzer *et al.*, 1988). However, the exact point of action of CR2 has not yet been established in the human system, although one would expect it to be similar to that described above. Human B-cell lines established from Burkitt's lymphoma or obtained by transformation of normal B cells with EBV *in vitro* are capable of growing in an autonomous fashion *in vitro*. It has been shown that autocrine growth factors produced by these cells contribute to their autonomous growth (Gordon *et al.*, 1984a,b; Blazar, Sutton, & Strome, 1983). Similarly, the inappropriate expression of some oncogenes is in some cases thought to render the cell independent of external stimuli and thus to bypass physiological control mechanisms (Rapp *et al.*, 1985; Kaczmarek *et al.*, 1985). In this study we employed a serum-free culture system to investigate whether autonomously growing Burkitt's lymphoma cell lines would still be susceptible to stimuli transmitted via CR2, whether human C3d would, under such conditions, stimulate human B cells and if the triggering of human B cells would occur via other epitopes than the binding site for C3d.

Abbreviations: C3, third component of complement; C3d, fragment of C3; CR2, C3d receptor; EBV, Epstein–Barr virus receptor; PMA, phorbol myristate acetate.

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MATERIAL AND METHODS

Cell culture

The human Burkitt lymphoma cell line, RAJI, was grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS),

glutamine, penicillin, streptomycin and fungizone. For this study the cells were adapted to serum-free culture by reducing the serum concentration to 5% for 3 days, then to 1% for another 3 days and then washing them three times in phosphate-buffered saline (PBS) and resuspending them in RPMI-1640 containing 0·25% bovine serum albumen (BSA) (Cohn Fraction V, No. A-4503 Sigma, St Louis, MO) as the only protein source.

For stimulation assays, RAJI cells adapted to serum-free culture for 1 week were cultured in 24-well plates or 96-well microtitre culture plates (Greiner) for 7 days, at a concentration of 1×10^5 cells/ml (1×10^5 or 2×10^4 cells/well, respectively) in the presence of several concentrations of purified human C3d or monoclonal antibodies. Antibodies and C3d were either present in solution or bound to the bottom of the culture wells. For binding to culture wells, 200 μl or 50 μl of PBS (10 mM Na phosphate, 140 mM NaCl) containing antibodies in the concentrations indicated in the test were added to a 1-ml or 200-μl culture well, respectively, and the plate incubated for 1 hr at room temperature. The contents were then aspirated and the wells saturated three times for 20 min at room temperature with 400 μl and 100 μl, respectively, of 0·25% BSA in RPMI-1640.

The cells were counted daily from Day 2 to 7 and cell viability was determined by trypan blue exclusion. Thymidine (TdR) incorporation was determined on Day 3 by pulsing the cells with 0·5 μCi [^{3}H]TdR/well for 4 hr. Stimulation experiments were always set up in triplicate.

Monoclonal antibodies to CR2

As reported previously (Petzer *et al.*, 1988), the mouse IgG1 anti-CR2 antibodies 1C8,2G7 and 6F7 are directed against three distinct epitopes localized on a 28,000 MW tryptic fragment of CR2. A fourth antibody, 1F8, recognizes the same epitope as 2G7. The 28,000 MW fragment is itself derived from the 72,000 MW tryptic fragment of CR2 carrying the binding site for C3d. Antibodies used in this study were purified from ascites by repeated precipitation (three times) with Na_2SO_4 followed by anionic ion-exchange chromatography on HPLC (LKB Bromma, 2133-100, Ultropac Column, TSK DEAE-3SW, 7·5 × 150 mm).

Purification of C3d

One litre of human plasma from outdated blood was incubated at 37° for 7 days in the presence of 0·1% NaN_3 and then fractionated by sequential precipitation with 3·5% and 15% polyethyleneglycol. The 15% pellet was redissolved in 10 mM Na-phosphate buffer, pH6·8 and applied to a DEAE-Sephacel column (Pharmacia) (gel bed 1 l). The column was washed extensively with running buffer and then developed with a 2 × 2·5 l gradient of 0–500 mM NaCl. Fractions containing C3 were identified by Ouchterlony and further analysed on Western blots stained with an antiserum to human C3d. Fractions containing the 43,000 MW C3dg and the 35,000 C3d MW band were pooled, concentrated and further purified by chromatography on a G-100 column (1 × 100 cm). Fractions containing C3dg and C3d were pooled separately.

Immunofluorescence and FACS analysis

Immunofluorescence with anti-CR2 antibodies was performed according to standard methods using a FITC-labelled goat anti-mouse IgG conjugate (Tago, Hamburg, FRG). In some experiments a biotinated monoclonal antibody to CR2 (Petzer *et al.*, 1988) was used in combination with streptavidin-conjugated phycoerythrin (Becton-Dickinson). Monoclonal antibody MAH-3, directed against factor H (Schulz *et al.*, 1984), was used as a control. Analysis by flow cytometry was done on a FACS III (Becton-Dickinson). Excitation was at 488 nm with an argon laser and emission was measured with band pass filter 530/30. The logarithmically amplified fluorescence signal is represented on a 120-channel scale covering about three decades. At least 10^4 cells were analysed in each experiment.

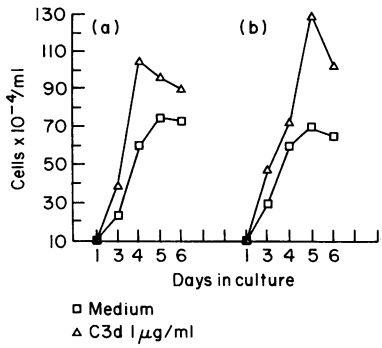

Figure 1. Enhancement of the growth of RAJI cells in serum-free culture by C3d. RAJI cells, adapted to serum-free culture as described in the Materials and Methods, were grown in the presence (△—△) or absence (□—□) of purified human C3d, which had been added to the culture (a) or attached to the bottom of the culture plate (b). Values shown are the means of triplicate wells and represent the result of one of three similar experiments. Variability within one experiment was generally less than 15%.

RESULTS

Purified C3d enhances the growth of RAJI cells in serum-free culture

RAJI cells were adapted to serum-free culture, as described in the Material and Methods, over a period of 7 days. We could keep RAJI cells inserum-free culture for up to 4 weeks with a viability between 80% and 90%. Under these conditions RAJI cells grew more slowly and reached a maximal culture density of 6–8 × 10^5 cells/ml (Fig. 1), i.e. about half the density obtained in serum-containing media. After 7 days in serum-free culture human C3d was added. In the presence of C3d RAJI cells reached a two to three-fold higher density (Fig. 1) and showed two to three times faster growth rate as assessed by TdR incorporation (Fig. 3).

This effect could be observed both with fluid-phase C3d and with C3d that had been bound previously to the bottom of the culture wells. However, C3d no longer enhanced the growth rate of RAJI cells in the presence of as little as 0·2% FCS.

Monoclonal antibodies to CR2 mimick the effect of C3d on the growth rate of RAJI cells

Culturing RAJI cells in the presence of several concentrations of monoclonal antibodies to three distinct epitopes on CR2 had a similar effect on the growth rate as well as the maximal culture density as did C3d. (Figs 2 and 3). An antibody to human IgM (anti-μ; clone 89) had no enhancing effect.

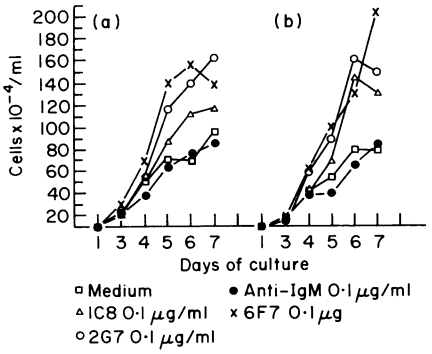

Figure 2. Enhancement of the growth of RAJI cells in serum-free culture by monoclonal antibodies to CR2. RAJI cells, adapted to serum-free cultures described in the Materials and Methods, were grown without (□—□) or in the presence of monoclonal antibodies to surface IgM (clone 89; ●—●) and three distinct epitopes of CR2: (△—△), clone 1C8; (○—○), clone 2G7; (×—×), clone 6F7. Antibodies were either added to the culture (a) or attached to the bottom of the culture plate (b). Values shown are the means of triplicate wells and represent the result of one of four similar experiments. Variability within one experiment was generally less than 15%.

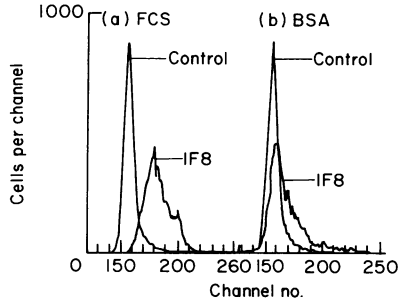

Figure 4. Expression of CR2 on RAJI cells grown in serum-free and in serum-containing culture. RAJI cells grown in serum-free culture (b) or in RPMI-medium containing 10% FCS (a) were stained in indirect immunofluorescence using monoclonal antibody 1F8, directed at the same epitope on CR2, which is recognized by 2G7 (Petzer *et al.*, 1988) and MAH-3 directed at complement factor H (MAH-3) as a control, and analysed by flow cytometry. Fluorescence intensity is displayed along the horizontal axis on a logarithmic 120 channel scale covering about three decades.

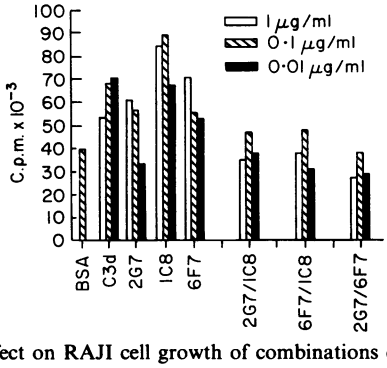

Figure 3. Effect on RAJI cell growth of combinations of monoclonal antibodies to CR2. RAJI cells in serum-free culture were grown in the presence of one single or pair-wise combinations of monoclonal antibodies to distinct epitopes of CR2 and their growth rate assessed by thymidine incorporation (0·5 μCi/well, 4-hr pulse) on Day 3. Shown are the means of triplicate samples, which varied by less than 10%. The figure represents one out of two similar experiments.

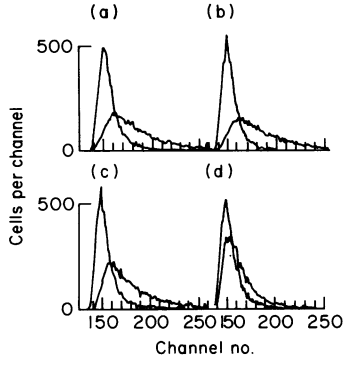

Figure 5. Expression of CR2 on RAJI cells is not up-regulated by ligands of CR2. RAJI cells were grown for 3 days in serum-free culture alone (a) or in the presence of stimulating concentrations of C3d (1 μg/ml; b), or monoclonal antibodies to CR2 2G7 (1 μg/ml; c) and 6F7 (1 μg/ml; d). Cells were harvested, washed and stained with biotinated 6F7 or biotinated monoclonal antibody to factor H (MAH-3) as a control, followed by avidin-conjugated phycoerythrin and analysed by flow-cytometry.

Simultaneous engagement of two epitopes on CR2 abolishes increased proliferation

The pair-wise addition of monoclonal antibodies to three different epitopes on CR2 does not induce the increase in proliferation that all three antibodies were able to induce on their own (Fig. 3). As single antibodies still exerted their stimulating effect in concentrations 10-fold higher than the concentrations used when two antibodies were added simultaneously, this observation cannot be explained by an overstimulation due to excessive doses of antibodies.

The expression of CR2 on RAJI cells is enhanced by a factor in FCS but not by C3d or monoclonal antibodies to CR2

Determining the effect of FCS on the quantitative expression of CR2 on RAJI cells we found that in the presence of serum RAJI cells expressed five to 10-fold more CR2 than in the absence of serum (Fig. 4). In order to test whether C3 or C3 fragments present in serum might induce a similar increase of CR2, we measured the expression of CR2 on RAJI cells cultured for 4 days in serum-free culture in the presence of stimulating concentrations of C3d or monoclonal antibodies to CR2. As shown in Fig. 5, neither C3d (b) nor a monoclonal stimulating antibody to CR2 (2G7, c) increased the expression of CR2 as measured in immunofluorescence using a biotinated antibody recognized a different epitope (6F7). The specificity of the fluorescence in this experiment is demonstrated in Fig. 5d where monoclonal antibody 6F7 (unconjugated) was used to stimulate RAJI cells which were then stained with the same antibody conjugated to biotin and avidin-conjugated phycoerythrin. Because antibody 6F7 (non-biotinated) bound to CR2 on the

cell surface during the culture the same antibody (in its biotinated form) stains RAJI cells only very weakly. These experiments would indicate that CR2 is most likely not up-regulated by its ligand but by another factor in FCS whose nature and mode of action is at present under investigation.

DISCUSSION

In this report we provide evidence that cells from the autonomously growing human Burkitt lymphoma cell line RAJI are still susceptible to control mechanisms exerted through the C3d/EBV receptor, CR2, whose engagement has been shown to promote the progression through the S-phase in the case of normal murine B cells (Melchers *et al.*, 1985). We also show that human C3d can stimulate human B cells under these conditions and that stimulation can occur via other epitopes on CR2 than the C3d-binding site.

We used a cell culture system that employed bovine serum albumin as the sole source of protein. The BSA used in this study was a commercially available Cohn V fraction that was not purified any further before use and could therefore still contain some impurities that might have been important for cell growth. In this cell culture system addition of purified C3d or C3dg as well as monoclonal antibodies to CR2 led to an enhanced proliferation, as demonstrated both by an increased TdR incorporation (Fig. 3) and an increase in cell number (Figs 1 and 2). Although the stimulatory effect of antibodies to CR2 has been noted previously by other authors (Nemerow *et al.*, 1985a; Frade *et al.*, 1985b) and ourselves (Petzer *et al.*, 1988), the experimental system used in this study offers the advantage of working with a homogenous cell population and therefore we can be certain that the observed stimulatory effects are not due to an indirect effect mediated by a small number of contaminating cells. The direct stimulatory effect of several ligands of CR2—C3 fragments as well as antibodies to various epitopes on CR2—on pre-activated B cells therefore seems firmly established.

Assuming a previously postulated model of the control of B-cell proliferation (Melchers *et al.*, 1985), one would speculate, on the basis of the results presented here, that the two 'restriction points' in the B-cell cycle, which are normally overcome by stimulation of the antigen receptor and by the action of a T-cell-derived growth factor, must be non-functional or bypassed on RAJI cells. Stimulation of B cells and T cells by mitogens, PMA, ionomycin or antibody to the CD3 molecule, i.e. agents that render the cell competent to respond to 'progression factors' (e.g. IL-2), is known to induce the expression of c-myc (Kelly *et al.*, 1983; Reed, Nowell & Hoover, 1985). RAJI cells are known to express c-myc (Maguire *et al.*, 1983) and it would be interesting to examine whether the expression of c-myc could be responsible for rendering a B cell responsive to stimulation via CR2. In addition RAJI cells produce an autocrine factor (Gordon *et al.*, 1984a, b; Blazar *et al.*, 1983) which could perhaps replace the progression factor normally provided by T cells.

Simultaneous use of two monoclonal antibodies to different epitopes of CR2 did not lead to the stimulation observed with either antibody alone. Whether this reflects the possibility that a particular way of cross-linking CR2 on the surface of the cell abrogates stimulation is unclear at present. In the case of the T11 antigen (CD2) it has been shown previously that only antibodies to certain epitopes produced a stimulation when used in combination, whereas others did not (Meuer *et al.*, 1984). RAJI cells grown in serum-containing media express five to 10-fold more CR2 than RAJI cells grown under serum-free conditions. This increase in CR2 does not seem to be induced by C3 present in culture media, as addition of C3d or of antibodies against CR2 to the serum-free culture did not enhance the expression of CR2. CR2 therefore does not seem to belong to the group of receptors that are up-regulated by their own ligand. The biochemical nature and the mode of action of the putative serum factor that induces the expression of CR2 is at present under investigation. While this study was in progress, other workers (Hatzfield *et al.*, 1988) have also noted the stimulatory effect of C3d on RAJI cells grown in serum-free culture.

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